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Resource-associated divergence in the arctic marine amphipod *Paramphithoe hystrix*

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Abstract Host specialization has played an important role in the speciation of groups such as herbivorous insects and parasitic invertebrates. In this study, we provide evidence for its role in the diversification of the amphipod *Paramphithoe hystrix* Ross 1835 in Arctic marine waters. This species lives and feeds on the tissues of varied invertebrate hosts that are much larger than itself, including poriferans, hydrozoans and echinoderms, acting as a 'micropredator'. We examined the genetic structure of *P. hystrix* at sites in the Canadian Arctic where it is represented by a white form found on the light-coloured sea star *Solaster endeca*, a red form on the pink soft coral *Gersemia rubriformis* and by a spotted form with an unidentified host. These phenotypes occur in microallopatry, sometimes occupying alternate hosts just a few metres apart. Although their variation in host associates might have arisen from ingested host tissue, our results indicate that these morphs are genetically distinct. Mitochondrial (cytochrome *c* oxidase I) and nuclear (28S ribosomal RNA) gene sequences, as well as allozyme data all show strong genetic divergence between the three colour morphs, demonstrating their reproductive isolation. These results suggest that *P. hystrix* is a complex of at least three species, which may have arisen as a result of disruptive selection following host switches. Moreover, the depths of genetic divergence indicate that diversification of this complex was complete prior to the Pleistocene.

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Introduction

Speciation as a result of host-race formation has produced some of the most diverse taxonomic assemblages in terrestrial as well as aquatic environments (reviewed in Tauber and Tauber 1989). The prevalence of resource-mediated speciation has been demonstrated most dramatically in phytophagous insects, which associate intimately with their host plants and are significantly more diverse than sister taxa with other feeding habits (Bush 1975a, b; Strong et al. 1984; Diehl and Bush 1989; Carroll and Boyd 1992). The potential role of resource specialization in marine arthropods has similarly been documented for several groups of commensal decapods. Stevens (1990) established that individuals of a pea crab displayed more genetic differentiation between sympatric individuals on different bivalve host species than between crabs on the same host from distant locations. Genetic differentiation related to high host specificity among sponge-dwelling alpheid shrimps similarly led Duffy (1992, 1993, 1996a, b) to conclude that substantial differentiation can be maintained despite some gene flow. Although arctic marine taxa have not been studied in detail, it appears that host-associations may be a common adaptation to resource limitations in the Arctic Ocean. For example, Vader (1978) found that species in 15 different amphipod families are associated with echinoderms in Norwegian waters. However, more detailed studies of these biological associations have not been undertaken. For instance, no investigation has yet considered the genetic consequences of differential host association in a polar species.

One reason for the limited scientific effort directed towards arctic diversity is the perception that evolutionary progression has been limited in this setting (reviewed by Thiel et al. 1996). Additionally, the Arctic is believed to contain a very young and impoverished fauna as a result of the devastating effects of the Pleistocene glaciations (Hewitt 2000). This

conclusion is congruent with the usual decline in species diversity with increased latitude (Rapaport's Rule, see Pianka 1966; Rohde 1992; Roy et al. 2000). However, a growing number of taxonomic groups, particularly in the south polar sea, appear exempt from this rule including nematodes, diatoms, bryozoans, polychaetes and amphipods (Sanders 1968; Clarke and Crame 1989; Barnard and Karaman 1991; Hillebrand and Azovsky 2001; Rex et al. 2001). Apparently, adaptations to polar conditions have enabled taxonomic and phylogenetic diversification in at least some taxa (Thiel et al. 1996). Moreover, adaptations to an environment of low temperatures and low productivity imply the emergence of novel evolutionary pathways. Therefore, information on diversification in polar life may provide valuable insights into evolutionary mechanisms at large when contrasted with the results from more intensively studied temperate and tropical systems.

Here, we present the first molecular survey investigating the role of resource-mediated differentiation within the common arctic marine amphipod *Paramphithoe hystrix* Ross 1835. A host association in this species was first noted by Stephensen (1935), who observed *P. hystrix,* as well as several allied species, living on deep-sea coral in European waters. Oshel and Steele (1985) later extended its host range by establishing that populations of *P. hystrix* off Newfoundland lived and fed on tissues of the sponge *Haliclona ventilabrum*, concluding that *P. hystrix* was a 'micropredator'. In the Canadian Arctic, we found *P. hystrix* on both the hydroid *Gersemia rubriformis* and on the asteroid *Solaster endeca*, adding a third phylum to the hosts utilized by it. Moreover, these varied host-associations had phenotypic correlates. Specimens of *P. hystrix* on *G. rubriformis* always possessed a bright to dark red body coloration, whereas individuals on *S. endeca* were always white. A third morph with a distinctive spotted (red and white) phenotype was also found, but its host association is uncertain.

In many cases, body colour variation in micropredators reflects phenotypic plasticity arising from the ingestion of host-tissue. Such background matching, which can occur without genetic divergence, is adaptive because it reduces the likelihood of detection by predators (Vader 1978; Martin and Britajev 1998). Alternatively, colour morphs can reflect the presence of unrecognized sibling species and in this case genetic divergence should be present at many gene loci, reflecting their reproductive isolation.

Here, we employ molecular analyses to test whether morphological diversity in *P. hystrix* reflects phenotypic plasticity or reproductive isolation. To test for genetic divergence between phenotypes from different hosts, we examined variation at seven allozyme loci as well as DNA-sequence data for a nuclear (28S ribosomal RNA) and a mitochondrial gene (cytochrome *c* oxidase I).

Materials and methods

Collection of samples

Specimens were collected from four locations in Nunavut, Canada, during three field seasons. Initial collections were made at Igloolik in 1996, but most samples were collected from Cornwallis Island in August 2000 and 2001 and from Igloolik, Somerset Island and Devon Island in August 2001 (Fig. 1). Sympatric populations of all three morphs, including just a single individual of the spotted form, were found at one location (Resolute Bay, Cornwallis Island). Two of the morphs were also collected at Igloolik (red in 1996, white in 2001), but the red form could not be genetically analysed due to poor preservation. Samples were obtained by both SCUBA and benthic drag from depths of less than 100 m, sorted in the field and either flash-frozen in liquid nitrogen or preserved in 90% ethanol for subsequent molecular analyses. Specimens were taxonomically confirmed as *Paramphithoe hystrix* using standard keys (Stephensen 1935; Barnard and Karaman 1991).

Allozyme electrophoresis

A total of 22 individuals from Resolute Bay, including from 1–13 representatives of each morph, were examined for allozyme variation at seven loci using cellulose acetate electrophoresis as described by Hebert and Beaton (1993). Tissue homogenates were prepared by grinding the terminal body segment of larger organisms (>7 mm), or the complete posterior half of smaller specimens (<7 mm) in 10–50 µl of distilled water. Enzymes screened included arginine kinase (APK, EC 2.7.3.3), glucose-6-phosphate isomerase (GPI, EC 5.3.1.8), phosphoglucomutase (PGM, EC 5.4.2.2), fumarate hydratase (FUM, EC 4.2.1.2), malate dehydrogenase (MDH-1, MDH-2, EC 1.1.1.37) and mannose-6-phosphate dehydrogenase (MPI, EC 5.3.1.8).

The allozyme data were analysed using Genetic Data Analysis (GDA) software (Lewis and Zaykin 1999). Genotypic frequencies were determined for each morph and polymorphic loci (0.99 criterion) were examined for deviations from Hardy-Weinberg equilibrium (HWE) using Fisher's exact test. Genetic distances among individuals were quantified using Nei's (1978) genetic identity.

DNA-sequence analysis

Genomic DNA was prepared using a modified proteinase K method (Schwenk 1996): Individual animals were homogenized in 50 µl of extraction buffer (10 mM Tris-HCl pH 8.3, 00.5 M potassium chloride, 0.005% Tween-20, 0.005% NP-40 and 20 µg of proteinase K) and incubated overnight in a 50°C oven. Following denaturation by incubation for 15 min in a 96°C water bath, extracts were stored at –20°C until analysed. The polymerase chain reaction was subsequently used to amplify a 658-bp fragment of the mitochondrial (mt) cytochrome *c* oxidase subunit I (COI) gene with the primer pair LCO1490 and HCO2198 (Folmer et al.1994), and a 727-bp fragment of the nuclear 28S rRNA gene using the primers 28F (5′-ccagctatcctgagggaacttcg-3′) and 28R (5′-gggactacccccctgaatttaagcat-3′). Each 50 µl PCR reaction consisted of 0.1–3 µl of genomic DNA template, 9 mM Tris HCl (pH 8.3), 45 mM KCl, 2.2 mM $MgCl_2$, 0.26 µM of each dNTP, 0.36 µM of each primer, and 0.7 U of *Taq* DNA polymerase. The thermal regime for amplification of both gene regions consisted of one cycle of 90 s at 94°C, 35 cycles of 45 s at 94°C, 60 s at 50°C, and 60 s at 72°C, followed by one cycle of 5 min at 72°C. The product was gel purified using the Qiaex II kit (Qiagen Inc.), subjected to dye terminator cycle-sequencing reactions (25 cycles with 55°C annealing temperature) with primer LCOI490 (COI) or 28SF (28S) and BigDye V3, and sequenced on an ABI 377 automated sequencer (Applied Biosystems).

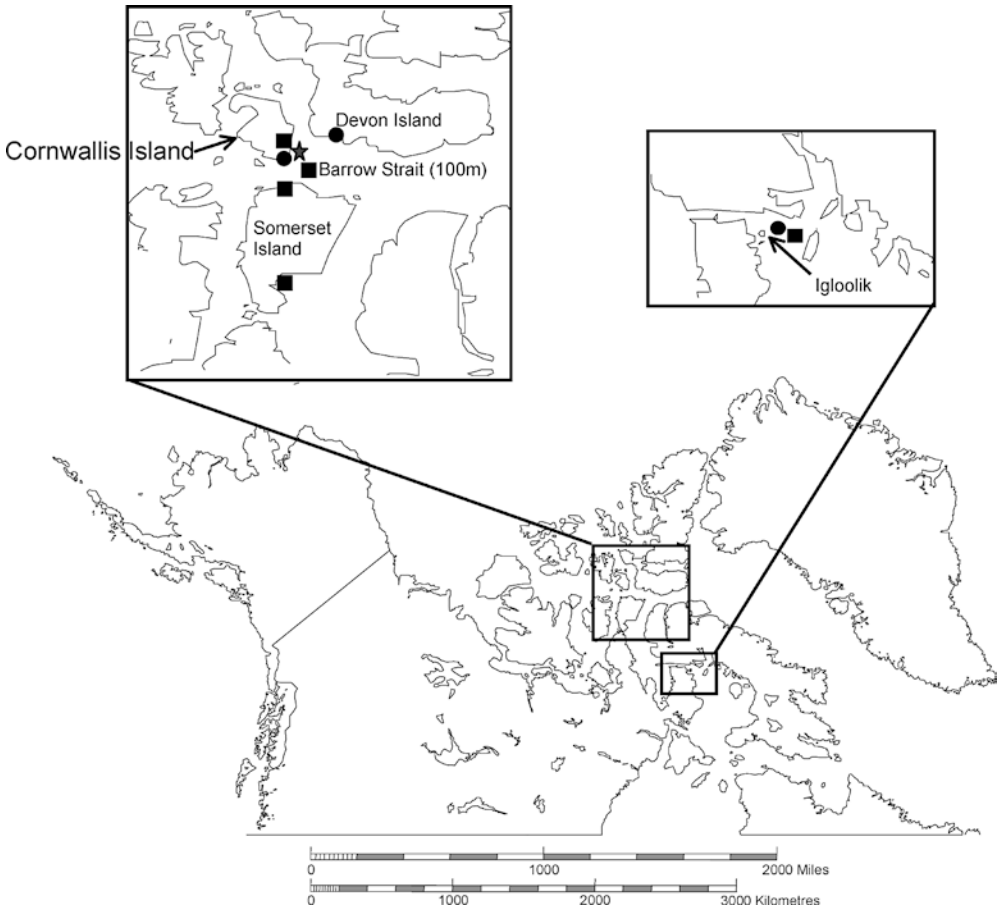

Fig. 1 Sampling localities for *Paramphithoe hystrix* in the Canadian Arctic showing the distribution of the three morphs: red (■), white (●) and spotted (★)

DNA sequences were edited and aligned using Sequencher (Gene Codes Corporation) and CLUSTAL X (Thompson et al. 1997). Closely related eusirid and epimerid amphipods of the genera *Eusirus*, *Rhachotropis* and *Epimeria* (GenBank accession number AF451341) were employed as outgroups for the phylogenetic analysis of COI. However, trees for the 28S sequence data were unrooted because the high incidence of indels compared to available outgroup taxa precluded a confident alignment. All sequences were deposited in GenBank with accession numbers AY271845–AY271854 for CO1 sequences and AY271830–AY271840 for 28S rRNA sequences.

A distance matrix of pairwise nucleotide sequence divergences was calculated using p-and Kimura-2-parameter distances in MEGA 2.1 (Kumar et al. 2001). All remaining analyses were conducted using the program PAUP*40b4 (Swofford 1998). A χ^2 goodness-of-fit test was performed on the sequence data for each gene region to determine if nucleotide composition was homogeneous among the taxa. Distance methods (UPGMA and NJ) were utilised to examine relationships among the genotypes detected at each locus. The stability of tree branches was assessed through bootstrap analysis based on 1,000 replicates.

Table 1 Gene frequencies at seven allozyme loci in three morphs of *Paramphitoe. hystrix* from Resolute Bay, Cornwallis Island

Loci		Red ($n=13$)	Spotted ($n=1$)	White ($n=7$)
APK	F	1.00	1.00	—
	S	—	—	1.00
PGI	F	0.47	—	—
	M	0.30	1.00	0.43
	S	0.17	—	0.57
	S′	0.07	—	—
PGM	F	—	0.50	1.00
	S	1.00	0.50	—
MPI	F	—	0.50	0.08
	S	1.00	0.50	0.92
MDH1	F	0.83	0.50	—
	S	0.17	0.50	1.00
MDH2	F	—	1.00	—
	S	1.00	—	1.00
FUM	S	1.00	N/A	1.00

Results

Allozyme variation

Six allozyme loci showed variation, whereas one locus (FUM) was monomorphic (Table 1). The mean percentage of polymorphic loci across all individuals was 35.7% (28.6–50%). Allelic diversity was relatively low with an average of 2.5 alleles per locus across polymorphic loci. When results from all individuals were pooled, each polymorphic locus showed a significant deviation from HWE ($P<0.015$), always due to heterozygote deficiency ($F=0.68$). The following diagnostic alleles were observed between the morphs: the white form possessed a diagnostic allele at the APK locus, while the spotted individual had a unique allele at

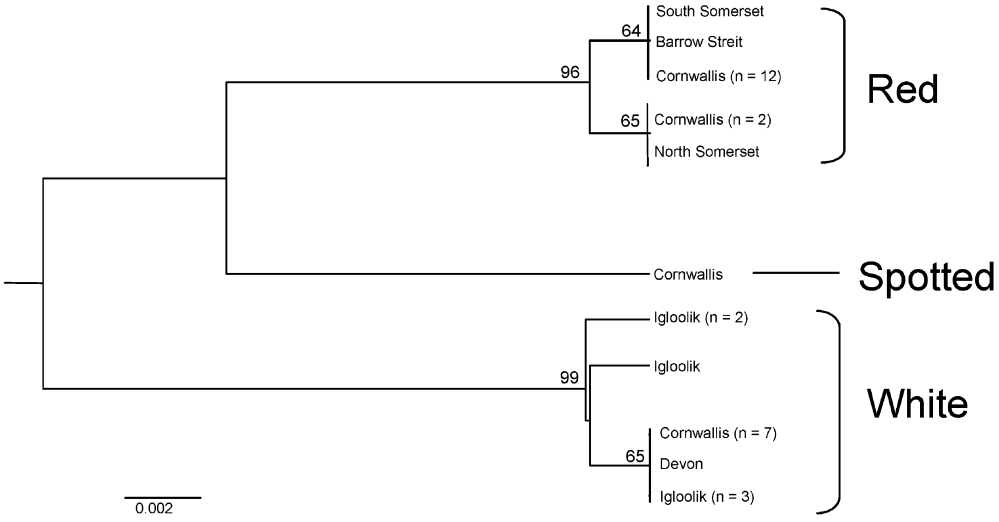

Fig. 2 UPGMA tree of 28S rRNA nucleotide variation (702 aligned sites) for the three morphs of *Paramphithoe hystrix* from five locations in the Canadian Arctic. Values above branches indicate bootstrap support of branch (1,000 replicates)

MDH-2 (Table 1). Although individuals of the red form lacked a diagnostic fixed difference at any single locus, all individuals of this species could be separated from the other two taxa by their diagnostic combination of alleles at the APK and MDH-2 loci. A pairwise comparison of Nei's (1978) genetic identity indicated marked allozyme divergence between the three phenotypes with the lowest divergence between the red and white forms (I = 0.42), followed closely by the white and the spotted forms (I = 0.48) and the highest between the spotted and the red forms (I = 0.73).

Nuclear sequence variation

Thirty-two individuals (17 red, 14 white, 1 spotted) were sequenced and aligned for a 702-bp region of the 28S rDNA gene. No sequence variation was detected at 671 nucleotide positions, but 31 sites were variable. All individuals of the white form could be distinguished by their possession of two diagnostic indels. Specifically, they showed two 1-bp insertions at positions 316 and 498, which were not shared by the red or the spotted forms. Distance analysis showed that the three colour morphs were separated into three distinct groups (Fig. 2). Sequence divergence among the colour morphs was as high as 3.45%, a much larger value than that within morph divergences which averaged 0.22% (Table 2), despite the fact that geographic distances between morphs were less than the distances between populations of the same morph (Fig. 1). A homogeneity test of base frequencies across all specimens revealed no significant differences in nucleotide composition for the rDNA sequences ($\chi^2 = 5.62$, $df = 93$, $P > 0.99$).

Table 2 Genetic distances (p-distance in %) among six haplotypes of *P. hystrix* based on the analysis of 702 bp of the 28S rRNA gene

	1.	2.	3.	4.	5.	6.
1. Cornwallis White	—					
2. Igoolik White	0.22	—				
3. Igloolik White	0.22	0.43	—			
4. Cornwallis Spotted	2.59	2.80	2.80	—		
5. Cornwallis Red	3.23	3.02	3.45	2.37	—	
6. Cornwallis Red	3.02	3.23	3.23	2.16	0.22	—

Mitochondrial sequence variation

Thirteen specimens, including a single individual of the white and spotted morphs, were sequenced and unambiguously aligned for a 632-nucleotide region of CO1. Efforts to sequence other white individuals failed. No indels were present and 548 sites were invariant while 84 were variable. As for the rDNA, the nucleotide composition of COI was homogeneous across taxa ($\chi^2 = 14.53$, $df = 45$, $P = 0.99$). Distance analysis indicated clear genetic divergence among the three morphs at COI (Fig. 3). However, sequence divergence at COI was distinctly higher between the morphs than at 28S rDNA with a maximum of 15.8% sequence divergence between the red and the white morph (Table 3). The spotted individual again shared more substitutions with the red morph than white morph (33 vs 23), but it also possessed 21 unique nucleotide changes. All except one of these substitutions occurred at the third position and none resulted in an amino acid substitution. By contrast, the white morph possessed five substitutions that were not at the third position and three led to an amino acid substitution.

Discussion

Both nuclear (allozyme, ribosomal DNA) and mitochondrial genes showed strong genetic differentiation among the morphs of *Paramphithoe hystrix*. Furthermore, fixed allozyme differences and diagnostic nucleotide substitutions support the conclusion that the three morphs are reproductively isolated. This conclusion was particularly strongly supported for the red and white

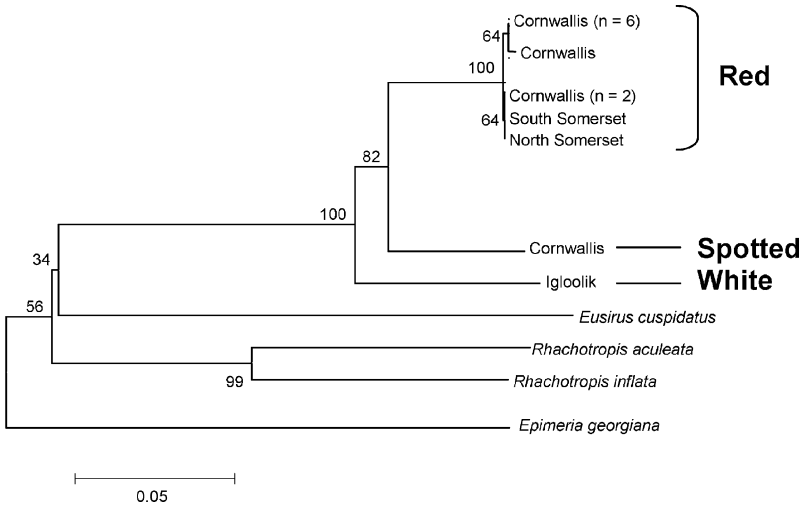

Fig. 3 Neighbor joining tree of CO1 nucleotide variation (632 aligned sites) for three morphs of *Paramphithoe hystrix* from three locations in the Canadian Arctic using the Tamura-Nei algorithm. The eusirid and epimerid amphipod genera *Eusirus, Rhachotropis* and *Epimeria* were used to root the tree. Values above branches indicate bootstrap support of branch (1,000 replicates)

Table 3 Genetic distance matrix for colour morphs of *P. hystrix* based on the analysis of 632 bp of the COI gene. Top diagonal p-distance (in %) between amino acid sequences of four haplotypes and an outgroup. Bottom diagonal shows % nucleotide divergences based on the Kimura-2-parameter model

	1	2	3	4	5
1. Igloolik White		2.2	2.2	2.2	11.6
2. Cornwallis Spotted	14.7		0.0	0.0	10.1
3. Somerset Red	15.8	12.0		0.0	10.1
4. Cornwallis Red	15.8	12.0	0.006		10.1
5. *Eusirus cuspidatus*	34.9	32.1	29.8	29.8	

morphs because intra-morph genetic divergence was far less than that between morphs. Furthermore, despite the analysis of just one spotted individual, its possession of diagnostic allozyme and sequence characters indicate that it is an independent lineage. This evidence for their reproductive isolation was reinforced by the presence of fixed allozyme differences between the morphs and genetic identity values ($I = 0.42$–0.73) that lie outside the usual within-species boundary (Stewart 1993; Hogg et al. 1999). This conclusion is further supported by the extent of CO1 sequence divergences among the morphs, which are similar to those present in other species assemblages of amphipods (Witt and Hebert 2000; Witt et al. 2003). Finally, differentiation at 28S rRNA indicates reproductive isolation between forms as divergences greater than 3.4% between the red and white forms lie well beyond the range of typical intraspecific divergences (Remigio and Hebert 2000). Hence, despite the small sample sizes, these multi-gene analyses provide convincing support that the three "morphs" are in fact different species.

The linkage of genetic divergences in *P. hystrix* to differential host-associations suggests that reproductive isolation may have evolved in microallopatry. Patchy host distributions and limited dispersal favour genetic fragmentation and increase the likelihood of reproductive isolation evolving within a small geographic scale (e.g. Bush 1975b; Strong et al. 1984; Knowlton 1993). Amphipods are good candidates for local divergence due to their brooding behaviour and non-planktonic larvae (Hogg et al. 1999 and references therein). Indeed, amphipods have been shown to rapidly accumulate genetic differences, particularly in species that occupy discrete habitats. However, little genetic divergence was observed in this study among populations of a single morph over large distances, indicating that low mobility is not the sole force promoting differentiation in this complex. The current maintenance of genetic divergence in microallopatry indicates that reproductive isolation is effectively complete. Whether this is chiefly due to differential host fidelity [stage 2, Berlocher (1998)] or whether prezygotic and/or postzygotic isolation have already evolved (stage 3) will require further work.

Numerous models have established that differential habitat preferences linked to weak assortative mating can lead to linkage disequilibria that foster the rapid evolution of reproductively isolated subpopulations (Solignac 1981, Doebeli 1996; Johnson et al. 1996; Dieckmann and Doebeli 1999; Kawata 2001). Using mathematical modelling, Kawata (2001) showed that assortative mating in conjunction with habitat-specific selection can maintain a stable resource polymorphism. Only slight phenological and ethological divergences are subsequently needed to produce reproductive isolation, such as that observed among sympatric populations of the marine isopod *Jaera albifrons* species complex (Solignac 1981). In the case of host associations, host specificity by sedentary adults can lead to mating on the host, creating ideal conditions for local differentiation. This sort of behaviour in a snapping shrimp species complex has apparently led to extensive radiation in sympatry (Duffy 1993, 1996b). Similarly, *P. hystrix* possesses ecological traits that would predispose it to diversification following a host switch. Adults show low mobility and brood their offspring, facilitating the occurrence of assortative mating linked to host use.

The present data cannot determine whether divergence in *P. hystrix* occurred in sympatry, allopatry, or parapatry as speciation occurred in the distant past. In fact, assuming that rates of molecular differentiation in *P. hystrix* are similar to those in the tropical snapping shrimp genus *Alpheus* (Knowlton et al. 1993), the three morphs of *P. hystrix* diverged some 6.7 million years to 8.8 million years ago. This result suggests that its diversification occurred well in advance of the onset of Pleistocene glaciations approximately two million years ago. Interestingly, the diversification of the North American bird fauna (Klicka and Zink 1997) was apparently largely complete some five million years ago, indicating that the conditions in advance of the Pleistocene did more to promote speciation of aquatic and terrestrial organisms than the glaciations themselves.

The cryptic divergence of *P. hystrix* revealed by this study confirms our limited knowledge of biotic diversity in the north and suggests that polar life merits more study. In fact, there is a growing recognition that polar environments have played a prominent role as centres of diversification for groups such as the amphipod and isopod crustaceans, which are dominated by coldwater taxa (Kussakin 1973; Wägele 1987; Barnard and Karaman 1991). The strength of this association is particularly evident in the amphipods where only 26 of 1,060 described genera are confined to warm shallow waters (Bernard and Karaman 1991). More comprehensive molecular work on such groups will undoubtedly clarify the origins and extent of arctic biodiversity. Moreover, by providing the information needed to contrast patterns of diversification in warm- and coldwater taxa, these studies will enable a broader perspective on mechanisms of diversification.

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